Review

Calpain inhibitors as potential treatment for stroke and other neurodegenerative diseases: recent trends and developments

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Calpain, a calcium-activated cysteine protease present in most mammalian tissues, including the brain, has been implicated in neurodegenerative processes resulting from its overactivation following cerebral ischaemia or traumatic injury to the head or spinal cord. Through significant effort, particularly over the past ten to fifteen years, the complex biochemistry and physiological roles of this important regulatory enzyme have been partially clarified. Despite remarkable advances in understanding calpain's normal functions, as well as its involvement in neuropathological conditions, a full appreciation of its role in neuronal cells remains elusive. A wide array of peptidic, peptide mimetic and non-peptide inhibitors have recently emerged, some of which display potency both *in vitro* and *in vivo* in various cell lines and animal models of focal and global ischaemia. A drug candidate has yet to be identified for advancement to clinical testing.

Keywords: *calpain, cysteine protease, ischaemia, neurodegeneration, stroke*

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1. Introduction - calpain characterisation and putative physiological roles

Calpain (EC 3.4.22.17) is a member of the cysteine protease class of enzymes, peptide-bond cleaving hydrolases featuring, by definition, a cysteine residue at the active site [1]. First described in 1964 [2], calpain actually comprises a group of homologous proteases (isozymes) which are found throughout the animal kingdom, including mammals, birds, molluscs and even insects [3-7]. The term calpain was introduced in 1981, alluding to its required activation by calcium and homology with papain [8].

At least six calpain isozymes have been identified thus far, of which several are apparently tissue-specific [9]. The two most common forms, however, are ubiquitous, found throughout most tissues in the body including the CNS, and differ from each other primarily in the concentration of calcium required for activation. Calpain I (μ-CANP) requires micromolar calcium concentrations (1 - 100 μM) whereas calpain II (m-CANP) is activated only at millimolar concentrations (0.1 - 1 mM) *in vitro*. However, significantly lower calcium levels are required for their activation in the presence of phospholipids (especially phosphotidylinositole-4,5-bisphosphate [PIP_2]) [10] and therefore the required calcium concentrations *in vivo* are likely to be commensurably lower. Calpains I and II are cytoplasmic, non-lysosomal heterodimers, composed of a 80 kDa catalytic and a 30 kDa regulatory subunit. The complete sequence of both subunits has been known since

1984 [11,12]. The calpains of a particular organism possess the same regulatory subunit while differences in the catalytic subunit account for their specificity and variable sensitivity to calcium [1]. Since calpains I and II are such closely related species, the general term calpain is used throughout this article to refer to both isozymes unless otherwise noted. Virtually all laboratories with an interest in drug discovery focus exclusively on calpain I, since it is the more calcium-sensitive variant and presumed to be more significant during episodes of excessive cellular calcium influx.

The 80 kDa catalytic subunit of calpain can be further divided into four domains, one of which (domain II) is somewhat homologous to other cysteine proteases (such as papain) especially over the regions comprising the catalytic residues, Cys^{108} and His^{265}. The function of domain III, which is not homologous to other known proteins, is unknown. Domain IV has four EF-hand Ca^{2+}-binding sites. The 30 kDa regulatory subunit has two domains. The N-terminal domain V appears to be important for interaction with phospholipids, which increase the enzyme's sensitivity to calcium. The C-terminal domain VI has another five EF-hand Ca^{2+}-binding sites, recently clarified by a crystal structure obtained of this domain complexed with PD 150606 (56, Warner-Lambert) [13]. It is unclear whether all or only some of the calcium-binding sites must be occupied for activation of the enzyme [11,14,15]. A number of reviews describe calpain's structure, apparent physiological roles and therapeutic potential [1,3,14,16-22].

Since cytosolic calcium levels are normally below 1 μM, it is thought that calpain generally exists in an inactive state *in vivo* (so-called procalpain), activated only when calcium concentrations reach threshold levels. Such behaviour would be consistent with signal transduction enzymes, which are generally quiescent until activated by some regulatory mechanism. Other factors, such as calmodulin and calpastatin, provide additional controls over calpain's activation and proteolytic activity [23,24]. The interplay of all these factors has complicated a full understanding of the timing and order of key events involved in the activation and proteolysis of calpain.

Calpain preferentially hydrolyses substrates with Leu or Val residues at the P_2 position, and a variety of amino acids at the P_1 position, though some secondary structure recognition may also play a role [25]. Most substrates feature some hydrophilic residues near the cleavage site, such as Pro, Glu, Asp, Ser, Thr, or short-lived PEST proteins (proteins with abundant Pro-Glu-Ser-Thr-rich sequences) [1,26].

Calpains I and II hydrolyse a diverse group of substrates. Along with the tight regulatory controls over its activity, this suggests calpain plays an important, though not yet fully defined, physiological role. Some known substrates include signal transduction regulators (such as protein kinase C [PKC] and other kinases), cytoskeletal proteins (for example, spectrin and MAP2), membrane-bound receptors (for example, epidermal growth factor), calmodulin-binding proteins, G-proteins and many transcription factors [10,14,27]. Along with the broad cell and tissue distribution observed, a rather wide range of possible physiological roles involving calpain have been inferred including mitosis, membrane fusion events, protein turnover, peptide hormone production, long-lasting synaptic modification and intracellular signalling [28,29].

Research over the past decade or so has gradually revealed at least some of calpain's specific physiological roles within the CNS and other organs. In contrast to the cathepsins, calpain proteolysis is generally limited in nature (rather than digestive), suggesting that it may serve as a biomodulator and signal transducing agent. Activation of protein kinase C (PKC) is one example of this. In the brain, calpain I appears to be associated primarily with dendrites and neuronal soma, whereas calpain II is present in axons and glia [30]. As mentioned earlier, calpain cleaves certain cytoskeletal proteins such as the microtubule-associated proteins (MAPs), spectrin (also known as fodrin or calspectrin), actin, lamin, tubulin and vimentin, prevalent structural proteins which are important for the integrity of neurones. Additionally, emerging evidence suggests an important role of calpain during neuronal differentiation of PC12 and SH-SY-5Y cells [16,31]. Calpain may play an important role in the adult nervous system during axonal sprouting, regeneration, and neurite extension and retraction [32]. Calpain may also alter synaptic strength within the brain and thereby participate in long-term memory function [33]. Significantly more research will be required before calpain's role in these and other processes can be fully delineated.

Owing to the wide scope of activity and virtual ubiquity of calpain throughout the body, a broad range of disease states has been potentially implicated following periods of acute or chronic calpain overactivation, presenting a host of opportunities for

pharmaceutical intervention. These include, most notably, cerebral ischaemia (stroke), brain trauma and spinal cord injury, subarachnoid haemorrhage, Alzheimer's disease, Parkinson's disease, muscular dystrophy, cardiac ischaemia, thrombotic platelet aggregation, restenosis, cataract, arthritis, and even cancer. Several reviews discuss these proposed areas in detail [14,18].

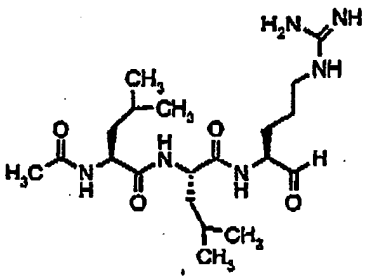
2. Rationale for the development of calpain inhibitors as potential therapy for ischaemic neurodegeneration

Cerebral ischaemia (stroke), traumatic brain injury, spinal cord injury and cardiac arrest lead to excessive levels of glutamate and increased intracellular calcium concentrations with subsequent activation of calpain and perhaps other calcium-dependent factors [34,35]. Most strokes are the result of a cerebral haemorrhage or an embolism originating either from the heart or an atherosclerotic arterial plaque which becomes lodged in a cerebral blood vessel. The resulting hypoxic, energy-starved neuronal cells lose their membrane potential, resulting in an uncontrolled release of neurotransmitters, notably the powerful excitatory neurotransmitter glutamate. This in turn leads to an overstimulation of the glutamate-associated NMDA receptors, which as one consequence causes calcium ions to flood into the neurones with concomitant overactivation of calpain (as well as other calcium-dependent systems such as calmodulin, PKC and phospholipase A_2 [PLA_2]). Subsequent rampant proteolysis of calpain substrates (cytoskeletal proteins, cell regulators and various membrane-associated receptors and channels) ultimately leads to an excitotoxic neurodegeneration and the overt symptoms resulting from the loss of associated functions, including weakness or paralysis, lost or slurred speech, ataxia, and even death [36,37]. Traumatic brain injury and spinal cord injury also display an aetiology similar to ischaemia, resulting in an excitotoxic necrosis which follows a two-phase neuropathologic progression. Following the initial damage as a result of the physical insult, a secondary neurodegenerative phase ensues, typically over the following one or two days and evidently tied to excessive glutamate and calcium concentrations.

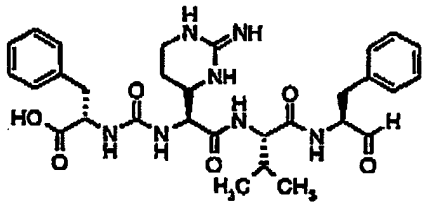
Several groups have clearly demonstrated that calpain activation promotes cell death in neuronal culture, brain slices and *in vivo* ischaemia models. Several reviews have recounted this pioneering research [14,18,38]. More recent reports [39,40] have continued to strengthen the prospect that calpain inhibitors may someday be one of the pharmaceutical options in a gradually evolving arsenal of drugs and medical techniques for the treatment of stroke and other neurodegenerative disorders, one of the leading causes of mortality in major industrialised countries. Some of the more significant aspects of this research, particularly with regard to stroke and traumatic brain injury or spinal cord injury, are discussed briefly below.

Studies conducted over the past ten to fifteen years have elucidated much of the biochemistry implicating interstitial glutamate with subsequent elevation of intracellular calcium and concomitant activation of calpain (and associated enzymes) as key initiators of neuronal necrosis [41,42]. Lynch and co-workers, for example, demonstrated an association of calpain proteolysis with neurodegeneration caused by neurotoxins, denervation, genetically impaired mice and perhaps even normal ageing [43]. A useful measure of calpain activity is the time-dependent proteolysis of spectrin (220 kDa) into characteristic fragments (150 kDa and 145 kDa) quantified by specific antibodies in response to an ischaemic event. Such breakdown products were readily observed in both *in vitro* (hippocampal slices) and *in vivo* models of ischaemia [44,45]. As mentioned earlier, calpain also processes other cytoskeletal proteins (e.g., MAP2), neurofilament proteins and plasma membranes. Unregulated metabolism of such key structural elements by calpain can lead to increased membrane permeability to ions and even macromolecules leading ultimately to neuronal death.

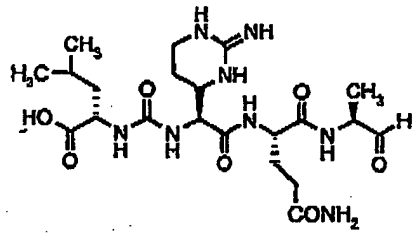
Evidence linking calpain activation to cell death in neuronal culture, brain slices, and *in vivo* models of ischaemia has been obtained from studies with calpain inhibitors. For example, in NaCN-induced hypoxia of chick embryonic neuronal cultures, necrosis was partially blocked by leupeptin (**1**, Ac-Leu-Leu-Arg-H) and calpain inhibitor I (**6**, Ac-Leu-Leu-Nle-H). Cerebellar Purkinje cells were also protected from AMPA toxicity by leupeptin, MDL 28170 (**8**, Merrell) or E-64 (**42**, a naturally occurring epoxysuccinate; see below) [46]. Wang and co-workers have also reported neuroprotection of Purkinje cells with PD 150606 (**56**, Warner-Lambert), a non-peptidic mercapto-acrylate (see below) [39]. However, such results may be equivocal. In cultured cerebellar granule cells, calpain inhibition (as



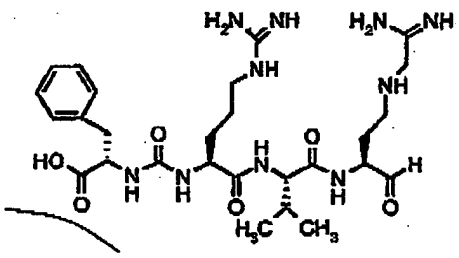
1 Leupeptin



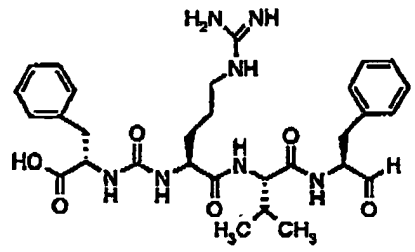
2 Chymostatin



3 Elastatinal



4 Antipain



5 β-MAPI

measured by spectrin breakdown inhibition) was not neuroprotective, suggesting a calpain-independent pathway in this cell line [47,48]. In a gerbil model of transient ischaemia, prolonged ventricular infusion of leupeptin (1) enhanced hippocampal CA1 neurone survival and reduced spectrin breakdown [49]. Similar results have also been reported in a rat model of focal ischaemia with calpain inhibitor I (6) [50] or MDL 28170 (8) [51]. Cerebral perfusion of AK275 (35, Alkermes), a ketoamide inhibitor (see below), reportedly reduced infarct size in a rat middle cerebral artery occlusion model [52]. Similar results were obtained with a close analogue, AK295 (36, Alkermes) [53,54]. Finally, researchers at Hoechst Marion Roussel have recently claimed a therapeutic window of opportunity of up to 6 h post middle cerebral artery occlusion in a focal cerebral ischaemia model in rats with iv bolus injections of MDL 28170 (8), determined through measurement of post-mortem infarct volume [40]. It is important to note, however, that many of the aldehydes and α-ketoamides utilised in the above investigations do not inhibit calpain with high selectivity, thereby leaving the results open to some interpretation.

3. Inhibitors

Enzyme inhibitors are classified generally as either reversible or irreversible, according to their mechanism of action. A reversible inhibitor is one which forms a non-covalent or labile covalent bond with the enzyme. Irreversible inhibitors, sometimes referred to as inactivators or suicide inhibitors, bind to the enzyme covalently, usually producing a leaving group as a by-product. Kinetic studies have been used to characterise inhibitors as reversible or irreversible (indistinguishable from slow-binding reversible). Alternatively, reversible inhibition can be demonstrated by restoration of enzymatic activity when the inhibitor concentration is decreased by dilution, gel filtration or dialysis. Several reviews provide a more in-depth discussion of enzyme inhibitor classes as they particularly relate to cysteine proteases [1,55].

Patent activity in the calpain field spans both reversible and irreversible inhibitors and it is still unclear which class is more likely to produce a therapeutically useful drug. A reversible inhibitor may arguably be a more likely drug candidate since its mode of action on calpain and other biomolecules might make it amenable for either chronic or

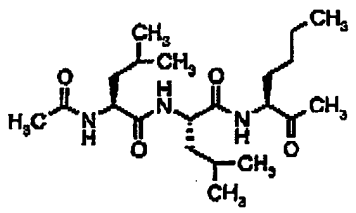
prophylactic administration. An irreversible inhibitor, on the other hand, could inactivate the entire pool of calpain, temporarily pre-empting the normal or essential physiological roles of this important but poorly understood biomodulator. Also, irreversible inhibitors may react with proteins to generate adducts that may be toxic or antigenic. Such obstacles notwithstanding, an irreversible inhibitor might offer a more suitable therapy during an acute, life-threatening episode of ischaemia or trauma, where completely inhibiting the enzyme may be critically essential and the most efficacious protocol.

3.1 Reversible inhibitors

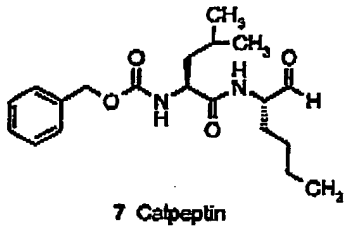
The design of reversible inhibitors for calpain (and cysteine or serine proteases in general) has been predicated on the tetrahedral intermediate produced by the enzymatic hydrolysis of an amide bond through nucleophilic attack on the carbonyl group by the sulfhydryl of the cysteine residue of the catalytic site (Cys^{108}). Such analogues often incorporate a carbonyl or carbonyl-like functionality which interacts at the active site. Additional peptidic, or peptide mimetic recognition elements, span the P and (in the case of non-aldehydes) P′ regions, and exploit hydrophobic, coulombic or hydrogen-bonding interactions to increase potency and selectivity. For calpain, optimal potency is generally achieved with di- or tripeptidyl analogues or corresponding mimetic congeners.

3.1.1 Aldehydes

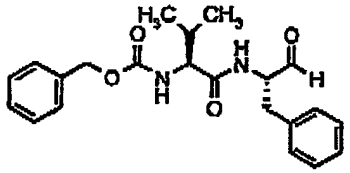
Peptidyl aldehydes were first identified as inhibitors of various cysteine and serine proteases through screening of culture filtrates of *Streptomyces* strains [56]. These closely related analogues, leupeptin (1), chymostatin (2), elastatinal (3), antipain (4) and β-MAPI (5), feature hydrophobic (Phe, Leu, Ala or Val) or basic (Arg, or cyclic guanidine) side-chains at P_1-P_4. Such inhibitors generally exhibit moderate potency at best and are not highly selective, however some selectivity *versus* the related cysteine proteases cathepsins B, H and L, may be achieved by variation of the P_1 or P_2 groups. A further shortcoming of these analogues is their inability to permeate the cell membrane due to the ionic charge of the Arg side-chains or terminal carboxylic acid groups. This subsequently led to neutral derivatives like calpeptin (7, Cbz-Leu-Nle-H) and MDL 28170 (8, Cbz-Val-Phe-H) [21,57-59,101,102]. Consistent with substrate preferences, Leu or Val is required at P_2 in these



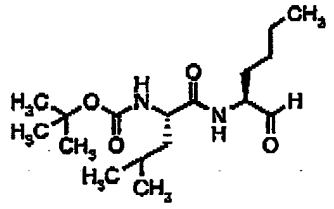
6 Calpain inhibitor I



7 Calpeptin



8 MDL 28170



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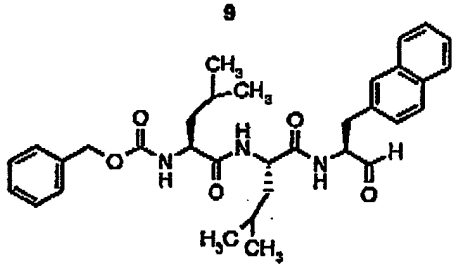
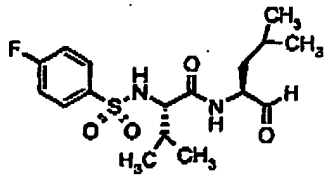


10 MG-121



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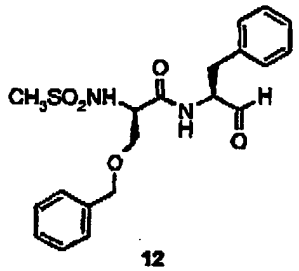

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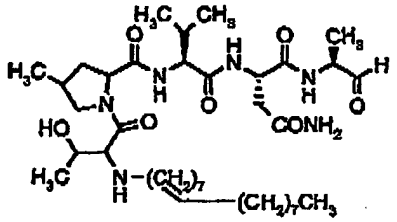

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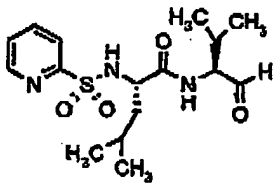

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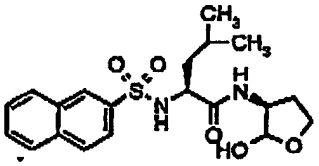

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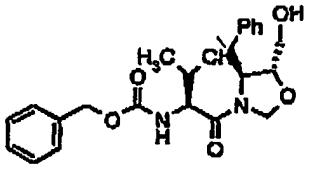

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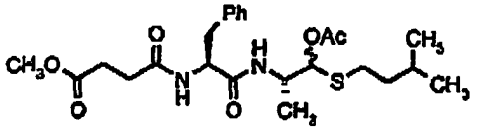

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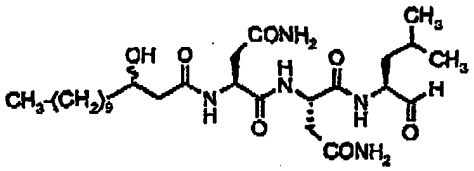
L,L-dipeptide aldehyde inhibitors. Suntory describes similar peptidyl aldehyde analogues such as **9** [60,103] and related derivatives, including acetals, oximes and semicarbazones as calpain (no biological data presented) and cathepsin L inhibitors [104]. Likewise, ProScript describes a series of peptidyl aldehydes incorporating unnatural amino acids or amide isosteres, specifically MG-121 (**10**), though no data are presented for calpain, their focus being primarily on 20S- and 26S-proteasome inhibitors [105].

As is readily apparent, researchers in this area have frequently capped the N-terminus with protecting groups frequently employed in synthetic chemistry, for example, Cbz and BOC. These prevent metabolism by aminopeptidases, eliminate the positively charged nitrogen (an impediment to cell penetration) that would otherwise exist at physiologic pH and provide an additional recognition element at the S_3 or S_4 subsites, which favour hydrophobic moieties. Senju has described dipeptidyl aldehydes with various aryl sulfonyl capping groups (e.g., **11**) as potent (8 nM) calpain inhibitors [106]. Also described in this application are the corresponding D-amino acids, although specific examples disclosed exhibit significantly less activity (4 - 100 μM).

Substantially more potent are Cephalon's P_2-O-alkyl-(or aryl-)D-serine class of peptide inhibitors in which an alkyl-(or aryl)sulfonamide group hypothetically occupies the S_2-pocket of the enzyme while an O-benzyl (or similar hydrophobic) group occupies the S_3 site. A specifically preferred compound (**12**) is a potent calpain inhibitor (8 nM) and four-fold selective against cathepsin B [61,107]. Cephalon has described pseudopeptides in which the P_3-P_2 amide groups were replaced by ketomethylene groups [108]. Fujisawa has described proline derivatives such as **13**, capped by hydrophobic alkanoyl groups, though no inhibitory data are disclosed [109]. A Mitsubishi Kasei Corporation US patent [110] describes various sulfonyl-capped dipeptidyl aldehydes, a particularly preferred example being **14** (6 nM). The casual observer might find some difficulty distinguishing between this and the Senju application [105] described above. The primary distinction between these two cases appears to hinge on a more restrictive genus for the P_2-P_1 residues in the Mitsubishi patent (exclusively Leu-Val-H) while Senju's claims for this region are more broadly inclusive of natural and unnatural hydrophobic amino acid residues. Conversely, Senju's descriptions for the capping group are restricted to 4-fluorobenzoyl,



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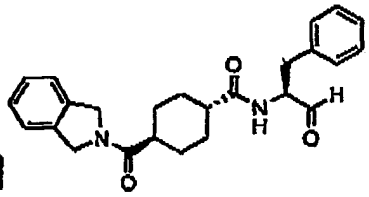

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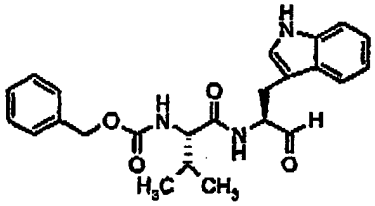

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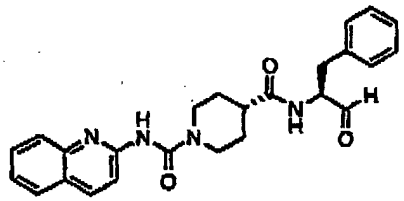

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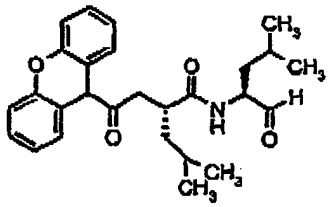

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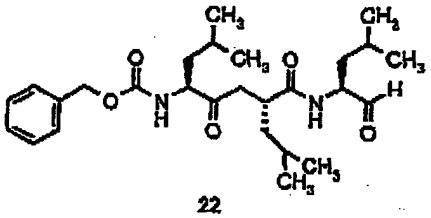

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4-chlorobenzoyl, 4-toluoyl and 2-naphthoyl, whereas for this region Mitsubishi rather broadly describes various substituted benzoyl, naphthoyl, and several heterocyclic acyl groups (e.g., pyridyl-, furyl-, isoquinolylcarbonyl).

Additionally, Mitsubishi has described sulfonyl (or acyl) capped analogues like **15** which also feature a cyclic hemiacetal (optionally O-acylated) formed through cyclisation of a P_1 homoserine residue with the aldehyde [111-113]. The strategy behind such 'masked' (prodrug) analogues is evidently to circumvent the inherent metabolic liabilities of aldehydes *in vivo* and perhaps also to address the longstanding challenges of developing compounds with the ability to permeate neuronal cells and cross the blood-brain barrier. Calpain inhibitory activity of preferred, non-O-acylated, examples is in the range of 30 - 110 nM *in vitro*. The inventors also describe liberation of the free hydroxyl analogues from the corresponding O-acetyl analogues following incubation in plasma, in support of their behaviour as prodrugs.

Another interesting application involving cyclic hemiacetals has been filed by Hoechst Marion Roussel [114] whereby dipeptidyl aldehydes are modified into 5-hydroxy-oxazolidine derivatives, like MDL 104903 (**16**), through cyclocondensation of the corresponding dipeptide with formaldehyde followed by reduction of the intermediate oxazolidine-5-one. Such analogues are claimed to be of nearly equal or, in some cases, even greater potency than their aldehyde counterparts. In particular, MDL 104903 exhibits nanomolar (33 nM) calpain inhibitory activity. The University of California has claimed O-acetylated thiohemiacetals as latent aldehyde prodrugs such as **17** [115]. Interest, however, appears to focus on the related cysteine proteases, cathepsins B, H and L, and only the weak activity of **17** against papain (3 µM) is reported. Taisho Pharmaceutical Company Ltd. has submitted two applications describing novel hydroxyalkanoyl capping groups, exemplified by **18** [116,117]. Such compounds are apparently modest (0.5 - 1.2 µM) calpain inhibitors. Takeda has three applications describing sulfonyl- or alkoxycarbonyl- (e.g., Cbz) capped peptide aldehydes like **19**, incorporating a tryptophan at P_1 [118-120]. These applications, however, are clearly aimed at cathepsins B and L (bone resorption therapy), as no biological data are presented for calpain.

Fuji Rebio has filed five closely related applications which generally feature a 1,4-disubstituted cyclohexane or piperidine scaffold as a P_2-P_3 peptide mimetic construct [121-125]. Such analogues (e.g., **20** and **21**) exhibit modest to good (40 - 400 nM) activity against calpain. Somewhat more potent are the

$\Psi[COCH_2]P_2$-P_3 analogues of Cephalon [126], as exemplified by **22** (12 nM) and **23** (25 nM) [62,63]. An additional example of the amenability of the P_2-P_3 amide group to peptide mimetic replacement is the focus of another Cephalon application which features a methylenesulfoxyl group as an amide bioisostere [127]. Analogues like **24** are remarkably good inhibitors (30 nM as a mixture of diastereomers) and display excellent selectivity *versus* serine proteases like thrombin and α-chymotrypsin [63,64]. In a very recent application Cephalon describes cyclic rigidified sulfonamides like 3,4-dihydrobenzothiazines and related heterocycles as inhibitors, conceptually derived from the O-benzyl-D-serines described above [128]. A specifically claimed example (**25**) is highly potent (6 nM) and interestingly, unlike **12** which disfavours N-substitution of the sulfonamide group, requires a small N-alkyl group on the sulfonamide to prevent hemiaminal formation from the aldehyde. Curiously, replacing the sulfonyl group with a carbonyl group leads to less active compounds. The 3,4-dihydrobenzothiazine analogues are about three to ten times more active than the corresponding benzothiazine, benzothiadiazine, dihydrobenzothiadiazine, or 4-hydroxybenzothiazine derivatives.

BASF has very recently described a series of aldehyde inhibitors in which the P_2 moiety has been replaced by aryl-substituted benzoyl groups (e.g., **26**) or other aryl-substituted benzoheterocycles; however, no potency data were presented [129-131].

Also very recently, SmithKline Beecham and Cephalon described a highly interesting series of aldehyde calpain inhibitors in which the P_2 amino acid was replaced by 2-substituted quinoline-4-carbonyl moieties [132]. The potency of the most active member of this series (**27**, 12 nM) compares very favorably with conventional L,L-dipeptide aldehydes. In addition, SmithKline Beecham very recently described a series of indole-2-carbonyl P_2 peptide-mimetic calpain inhibitor aldehydes, one of which (**28**) is quite potent (30 nM) [133]. The discovery by Cephalon and SmithKline Beecham of potent inhibitors bearing benzothiazines, indoles, and quinolines at P_2 is the clearest demonstration so far that peptide mimetic constructs can replace Leu or Val at P_2.

Though not bearing directly on calpain as such, Athena Neurosciences, Inc. has disclosed a broad range of alkoxycarbonyl capped mono-, di- and tripeptide aldehydes as cathepsin inhibitors [134], specifically cathepsin Y, as a method for inhibiting the secretion of β-amyloid peptide for the treatment of Alzheimer's disease.

Over the years, calpains have typically been isolated from a number of sources under differing conditions for studies against its various substrates and inhibitors [65]. Consequently, some inconsistencies emerged with respect to measured IC_{50} values, even with respect to the rank order of potency for standard inhibitors. Recombinant human calpain I has been prepared recently, providing a standardised source of the enzyme [66]. Cephalon researchers have used this recombinant calpain to reconcile the inconsistencies for peptide aldehydes, the most commonly studied class of reversible inhibitor [59]. This has helped to establish a more reliable foundation of subsite requirements enroute to developing a more accurate pharmacophore map and ultimately more potent inhibitors.

3.1.2 Peptide ketones

Mitsubishi Kasei made the interesting discovery that peptidyl cyclopropenone alcohols (**29**, $IC_{50} = 350$ nM) are fairly potent inhibitors of cysteine proteases, including calpain [67,135]. The inhibitory mechanism of this class of compounds has not yet been elucidated. One possibility involves protonation of the carbonyl oxygen to generate a relatively stable 2π-aromatic cationic system. Alternatively (or perhaps in tandem), the active-site cysteine sulfhydryl group may react nucleophilically with the carbon of the carbonyl group or either alkene carbon. The alcohol functionality may also be involved as a hydrogen bond donor or acceptor.

Unactivated peptidyl ketones exhibit very weak calpain inhibitory activity [68]. For example, tripeptidyl heteroaryl alkyl ketones described by Mitsubishi Kasei are weak calpain inhibitors [136]. In contrast to their excellent potency as serine protease inhibitors, trifluoromethylketones are only weak inhibitors of cysteine proteases [71], including calpain [68].

Cephalon has described reversible α-ketophosphorous-containing inhibitors, whereby the ester group of an α-ketoester (analogues discussed in the following section) is replaced by an appropriate phosphorous isostere with the objective of retaining or even increasing the electrophilic character of the α-keto functionality towards nucleophilic attack by the sulfhydryl group of the active site cysteine [137]. Some analogues do indeed display

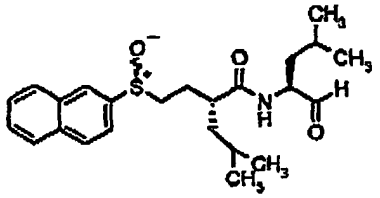
comparable activity to the corresponding α-ketoesters (e.g., **30**, 430 nM *vs.* Cbz-Leu-Leu-CO_2Et, 600 nM), however a divergence of SAR was observed for larger alkyl ester groups [69]. Surprisingly, despite the general observation that α-ketocarboxylic acids are significantly more potent than their corresponding esters, the monomethyl α-ketophosphonate of **30** is far less active (5 μM), in sharp contrast to the corresponding α-ketocarboxylic acid (Cbz-Leu-Leu-CO_2H, 7 nM).

A further attempt to develop activated ketones prompted the design of peptidyl heterocycles like **31**, but these were found to be weakly active inhibitors at best (~ 6 μM) [70]. Evidently, the potency observed with α-ketocarboxylic acid derivatives derives from more than simply the electrophilicity of the α-keto group.

3.1.3 α-Dicarbonyl analogues

As mentioned earlier, peptidyl aldehydes generally display poor selectivity towards various cysteine and serine proteases. It is therefore difficult to attribute their activity in cell cultures or animals to inhibition of any one specific enzyme [71]. Furthermore, their anticipated metabolic instability may present additional significant hurdles against such analogues ever progressing into clinical trials. Since methyl ketone, trifluoromethyl ketone (TFMK), cyclopropenone, nitrile, semicarbazone or phosphorous-based groups lack significant inhibitory potency towards cysteine proteases, other electron attracting groups have been investigated as potentially suitable alternatives. The most promising inhibitors reported thus far are α-dicarbonyl derivatives, especially the α-ketocarboxylic acid analogues, originally developed as inhibitors of serine proteases and aminopeptidases [72-75].

Investigators at Merrell Dow and Georgia Institute of Technology discovered that while peptidyl α-keto-esters were only moderately potent calpain inhibitors, α-ketoamides and α-ketoacids are quite potent [73,76,77]. A series of closely related patents and applications, filed by Georgia Tech Research Corporation [138-141] in partial collaboration with Cortex Pharmaceuticals, Inc. [142,143], describe a broad range of peptidyl α-ketocarboxylic acid derivatives, especially α-ketoamides such as **32** (15 nM). Benzyloxycarbonyl-capped dipeptides and other less common capping groups (e.g., other alkoxycarbonyl, aminocarbonyl, alkanoyl) are claimed. Leucine is



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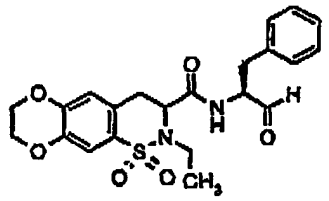

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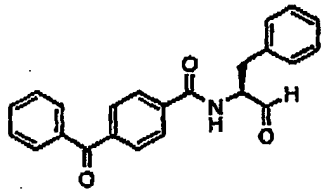

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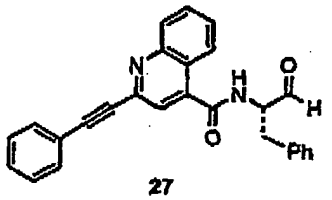

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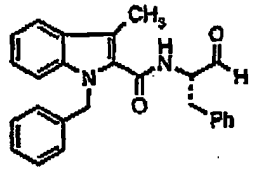

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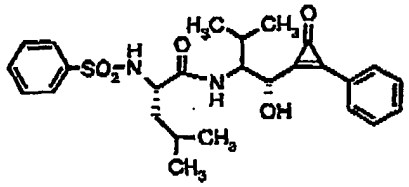

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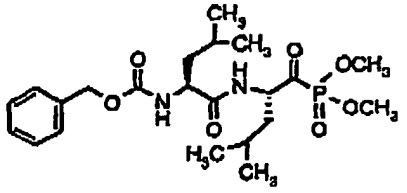

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Table 1. Reversible calpain inhibitors.

Compound	Calpain I IC_{50} (nM)	[Ref]
8 MDL 28170	10	[129]
9	30	[129]
11	7	[129]
12	5	[129]
14	6	[129]
15	32	[129]
16 MDL 104963	30	[129]
18	10	[129]
20	60	[129]
21	42	[129]
22	42	[129]
23	2	[129]
24	30	[129]
25	6	[129]
27	12	[132]
28	30	[132]
29	350	[132]
30	50	[132]
31	6000	[132]
32	15	[132]
33	31	[132]
34	129	[132]
35 AK-275	70	[132]
36 AK-295	32	[132]
37	20	[132]
38	77	[132]
55	10,000	[131]
56 PD 150606	210	[131]
57	50	[131]
58	6500	[131]
59	2700	[131]
Aminocarboxylic acid	22,000	[92]

most commonly employed at P_2 while Abu, Nle, Phe, Ala or less commonly Nva, Nle or Lys are claimed at P_1 (including D, L or racemic mixtures). The US patents issued in 1995 and 1997 generally involve variation of the peptidyl regions while the remaining applications cover the P' substituents of the α-ketoamide group.

Alkermes, Inc. has also described a series of peptidyl α-ketoamides, wherein the P' substituent is derived from an amino acid or aminosulfone derivative [78,144]. The inventors claim L, D or racemic mixtures at P_1 and P_2, though emphasis is on the L-configuration. Several disclosed examples (**33**, **34**) are fairly active calpain inhibitors (31 nM, 129 nM, res.). The pharmacologically interesting α-ketoamides AK-275 (**35**, 77 nM) and AK-295 (**36**, 32 nM), which are active *in vivo*, are also covered by this patent. Capping groups for which biological data are presented are exclusively Cbz or BOC, however, other more polar groups such as morpholinocarbonyl and dimethylaminocarbonyl are also disclosed. Presumably, by combining moderately polar groups at both the C- and N-termini the inventors hoped to improve solubility while maintaining adequate potency. The absence of biological data for any such analogues, however, suggests such compounds are probably only weak calpain inhibitors.

Cephalon's benzothiazine application described above for the aldehyde class also cover α-ketocarboxylic acid derivatives [128], particularly α-ketoamides like **37** (20 nM). Like their aldehyde counterparts, the 3,4-dihydrobenzothiazines are significantly more active than the corresponding benzothiazines, benzothiadiazines, or 4-hydroxybenzothiazines.

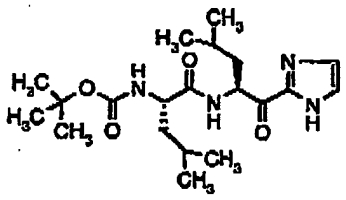
SmithKline Beecham and Cephalon have likewise described a relatively potent P_2 quinoline-4-carbonyl α-ketoamide (**38**, 77 nM), an analogue of aldehyde **27** [132].

BASF has described a series of α-ketoamide calpain inhibitors in which the P_2 moiety has been replaced by an N-acylated piperidine-4-carboxylate construct (e.g., **39**) [145] or by aryl- or heteroaryl-substituted benzoyl groups (e.g., **40**) [130,146]. However, no potency data were reported. Suntory has described α- (and β-) ketoesters like **41** as thiol protease inhibitors as part of their application on aldehyde derivatives discussed above [104]. Biological data, however, were reported only for cathepsin L.

Table 1 lists some representative reversible inhibitor analogues and their activities against isolated calpain.

3.2 Irreversible inhibitors

The following calpain inhibitors are classified as irreversible most frequently on the basis of time-dependent enzyme inhibition kinetics. In a few cases, stable covalent adducts with calpain or other cysteine proteases have actually been characterised.



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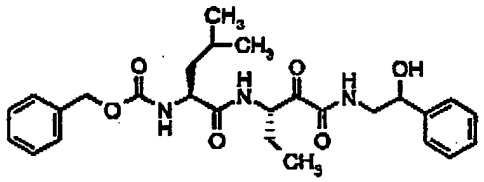

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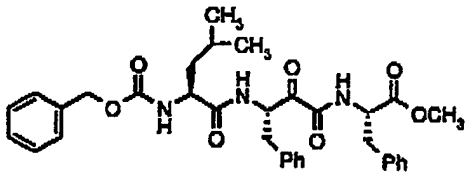

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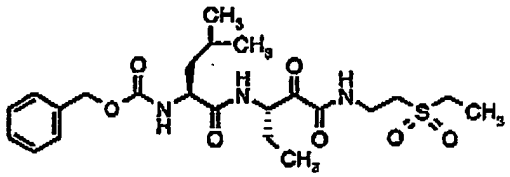

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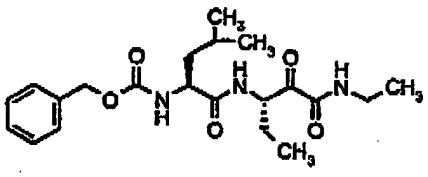
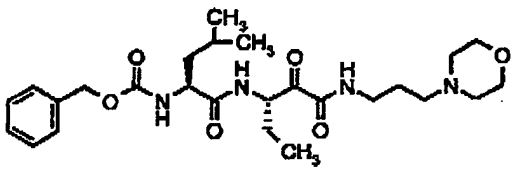
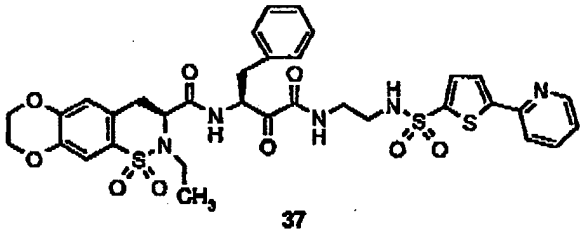


35 AK275



35 AK295



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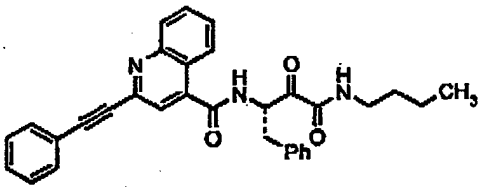

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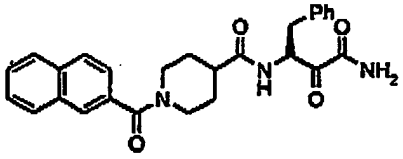

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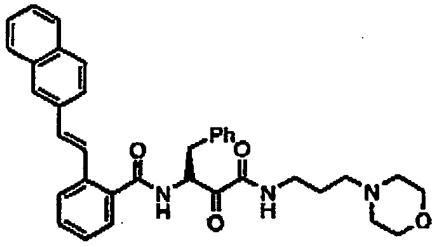

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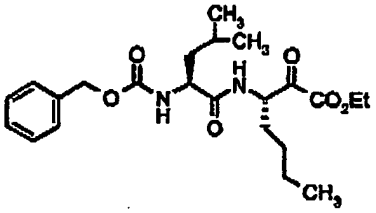

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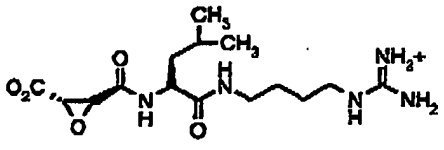


42 E-64

Table 2. Irreversible calpain inhibitors.

Compound	Calpain inactivation rate ($M^{-1}s^{-1}$)	[Ref.]
42, E-64	7,500	[79,80]
43, Ep-460	23,000	[66,80]
44, E-64c, Ep-475	2,450	[81]
Cbz-Leu-Leu-Leu-CH=N-	200	[84]
Cbz-Leu-Leu-Tyr-CH=N-	1,470	[84]
Cbz-Leu-Leu-Phe-CH=N-	500	[66]
Cbz-Leu-Leu-Tyr-CH=N-	230,000	[84]
Cbz-Leu-Gly-CH$_2$Cl	3,000	[65]
Cbz-Leu-Phe-CH$_2$Cl	5,500	[65]
Cbz-Leu-Leu-Phe-CH$_2$Cl	9,500	[85]
Leu-Leu-Phe-CH$_2$Cl	545	[85]
46	109	[150]
Cbz-Leu-Tyr-CH$_2$F	7,800	[86]
Cbz-Leu-Leu-Tyr-CH$_2$F	28,900	[86]
Cbz-Leu-Phe-CH$_2$F	136,100	[87]
47	375,000	[87]
48	290,000	[87]
49	5,600	[108]
50	26,000	[107]
51	230,000	[151]
Cbz-Leu-Leu-Phe-CH$_2$-S-(CH$_2$)$_2$-Ph	200,000	[90]
Cbz-Leu-Phe-CH$_2$-O-(2,6-difluoro-benzyl)	18,000	[166,90]
Cbz-Leu-Phe-CH$_2$-N(CH$_3$)$_2$	700	[166]
Cbz-Leu-Phe-CH$_2$-OP(=O)(Ph)$_2$	10,000	[89]
52	365,000	[137]
53	3,500	[154]
54	28,300	[91,155]
Phe-Gln-Val-Val-Gly-(3-NO$_2$-2-pyridyl-S)-Gly-NH$_2$	5,850	[93]

Second order inactivation rate constants were generally corrected for substrate concentration and reported as $k_{obs}/[I]$ or $k_{obs}/([I] + [S]/K_m)$.

3.2.1 Epoxysuccinates

E-64 (**42**), a naturally occurring epoxysuccinate cysteine protease inhibitor, was isolated from *Aspergillus japonicus* [79], and several naturally occurring analogues were subsequently discovered. Investigators at Taisho and elsewhere have prepared a large number of synthetic analogues of E-64 in which the guanidino functionality was modified and the amino acid was varied [106,147]. Mechanistically, the active-site cysteine is irreversibly alkylated by the reactive epoxide group. As a class, the epoxysuccinates generally inhibit other cysteine proteases more rapidly than they inhibit calpain. At best, epoxysuccinates display only moderate reactivity towards calpain (Ep-460, **43**, second order rate constant = 23,000 $M^{-1}s^{-1}$; see **Table 2**) [66,80]. The epoxysuccinate E-64c (**44**) decreases degradation of cerebral MAP2 in the rat MCAO model [80]. Epoxysuccinate esters possessing improved membrane permeability have been examined as prodrugs and display efficacy in dystrophic animal models, but were ineffective in treating human Duchenne muscular dystrophy [82]. The ethyl ester of E-64c, loxistatin (E-64d, Taisho), is hepatotoxic to rats [83]. Takeda has described aziridine analogues [148] and Japan Tobacco has

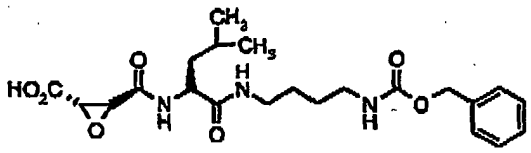
claimed dioxathiolane analogues (e.g., **45**) of epoxysuccinates [149].

3.2.2 Halomethyl-, diazomethyl- and acyloxymethyl ketones and related irreversible inhibitors

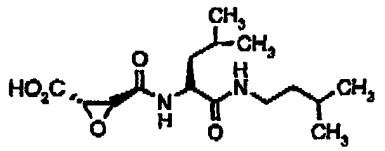
Halomethyl ketones and diazomethyl ketones are irreversible inhibitors of various cysteine proteases [71]. Tripeptide diazomethyl ketones such as Cbz-Leu-Leu-Tyr-CH=N_2 (230,000 $M^{-1}s^{-1}$) are potent irreversible calpain inhibitors [84], while dipeptide diazomethyl ketones such as Cbz-Leu-Phe-CH=N_2 (1,500 $M^{-1}s^{-1}$) and Cbz-Leu-Tyr-CH=N_2 (1,470 $M^{-1}s^{-1}$) are much less potent [66,84]. Chloromethyl ketones, including Cbz-Leu-Gly-CH_2Cl (31,000 $M^{-1}s^{-1}$), Cbz-Leu-Phe-CH_2Cl (5,500 $M^{-1}s^{-1}$) and Cbz-Leu-Leu-Phe-CH_2Cl (9,500 $M^{-1}s^{-1}$) are moderately potent irreversible calpain inhibitors [65,85,102,103]. Warner-Lambert has described a weak chloromethyl ketone calpain inhibitor (**46**) with a non-peptidic scaffold [150].

There is concern that the promiscuous reactivity of diazomethyl ketones and chloromethyl ketones toward a variety of endogenous nucleophiles could lead to undesirable inhibition of numerous non-target enzymes and cause toxicity. Accordingly, considerable effort has been directed at the discovery of analogues with less reactive leaving groups [55]. Cbz-capped di- and tripeptidyl fluoromethyl ketones Cbz-Leu-Tyr-CH_2F (17,000 $M^{-1}s^{-1}$) and Cbz-Leu-Leu-Tyr-CH_2F (28,900 $M^{-1}s^{-1}$) are moderately potent calpain inhibitors [86]. A dipeptidyl fluoromethyl ketone (**47**, 276,000 $M^{-1}s^{-1}$) and the tripeptidyl analogue Cbz-Leu-Leu-Phe-CH_2F (**48**, 290,000 $M^{-1}s^{-1}$), however, are significantly more potent [87]. Covalent stoichiometric binding was confirmed with radiolabelled **48**, which was found to be cell-permeable and to inhibit intracellular calpain. Cephalon has described P_2-P_3 ketomethylene (**49**, 75,600 $M^{-1}s^{-1}$) pseudopeptide and P_2-D (**50**, 26,600 $M^{-1}s^{-1}$) fluoromethyl ketone calpain inhibitors [107,108]. No overt toxicity was observed when the cathepsin B inhibitor Cbz-Phe-Ala-CH_2F was administered orally to rats, suggesting that the fluoromethyl ketone enzyme-reactive group may be relatively non-toxic [88].

Cephalon has described benzotriazolyloxy methyl ketones (**51**, 230,000 $M^{-1}s^{-1}$) and benzotriazin-4-one-3-yl-oxy methyl ketones as particularly potent irreversible calpain inhibitors [151]. Prototek has



43 Ep-460



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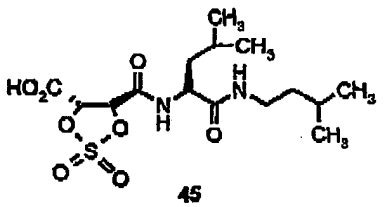

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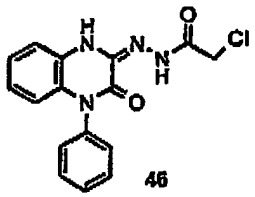

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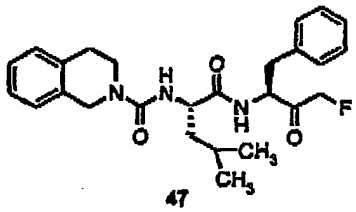

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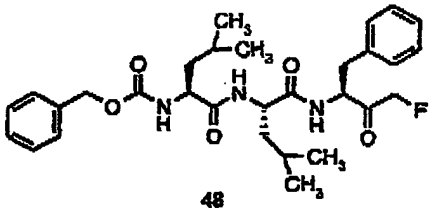

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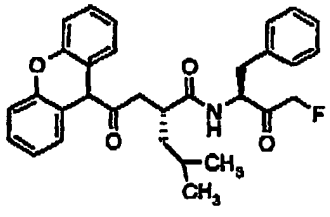

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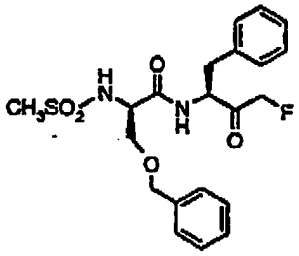

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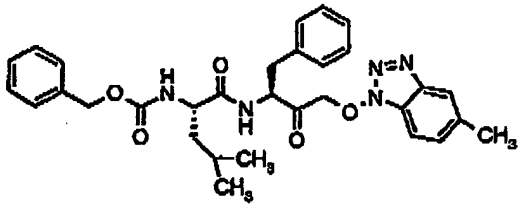

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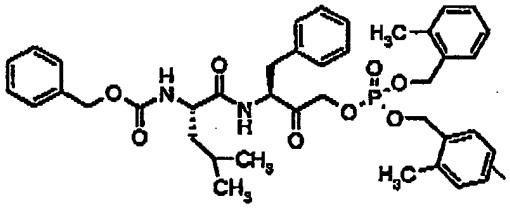

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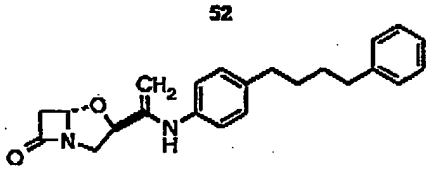

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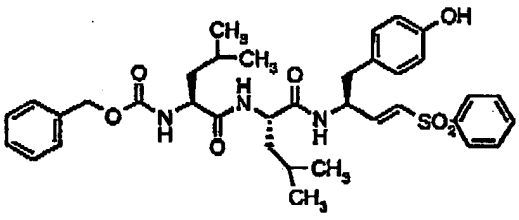

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described a series of aryloxymethyl ketones and heteroaryloxymethyl ketones (i.e., pyridyloxy-, pyrimidinyloxy-, and 2-oxodihydrofuran-4-yl-oxy methyl ketones) as inhibitors of cysteine proteases, but rates of calpain inhibition were not presented [152].

Certain dipeptidyl methyl phosphinates, phosphonates, and phosphates described by Sterling Winthrop [89] and Cephalon [137] are extremely reactive calpain inhibitors (52, 365,000 $M^{-1}s^{-1}$). In contrast, dipeptidyl acyloxymethyl ketones, which are highly reactive inhibitors of other cysteine proteases are only moderately potent calpain inhibitors (i.e., Cbz-Leu-Phe-CH_2O-(2,6-dichlorobenzoate); 19,000 $M^{-1}s^{-1}$) and aryloxymethyl ketones are almost inactive [66,89,90,102]. However, a tripeptide dimethylsulfonium methyl ketone is an extremely reactive calpain inhibitor [90].

Mitsubishi has prepared a large number of alkoxy, alkylthio, and dialkylamino methyl ketones which are weak calpain inhibitors [153]. Investigators at Cephalon determined that the dimethylamino methyl ketone Cbz-Leu-Phe-$CH_2N(CH_3)_2$ is a weak irreversible inhibitor (1,700 $M^{-1}s^{-1}$) [66]. The low reactivity of these inhibitors may reflect poor leaving group ability, but surprisingly, several rapidly inhibit papain, cathepsin B, and cathepsin L [153].

SmithKline Beecham has described a series of clavam-3-carboxamide derivatives which irreversibly inhibit calpain [154]. The most reactive of these, (phenylbutyl)phenyl amide 53, though only moderately potent (23,500 $M^{-1}s^{-1}$), is intriguing because it completely lacks the peptidic recognition elements usually required for strong binding to calpain. The activity of these clavams as calpain inhibitors constitutes one of the few examples of β-lactam cysteine protease inhibitors. β-Lactams are much better represented as inhibitors of serine proteases and other enzymes bearing active-site serine residues.

3.2.3 Michael acceptors

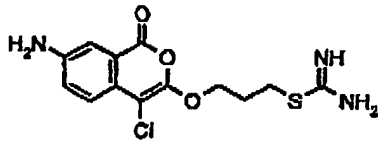
Investigators at Kephri have reported that tripeptide vinyl sulfone Michael acceptors like 54 are moderately reactive irreversible calpain inhibitors. Dipeptide vinyl sulfones are poor inhibitors of calpain, but highly reactive toward other cysteine proteases [91,155].

Table 2 lists representative irreversible calpain inhibitors along with their observed second order rates of inactivation.

4. Miscellaneous inhibitors

Several classes of calpain inhibitors, primarily non-peptidic, do not fall clearly into the series of compounds described above. Most are significantly less potent than the peptidyl analogues or peptide mimetics heretofore discussed. For example, Georgia

Tech Research Corporation and Cortex describe isocoumarin analogues with basic substituents as calpain inhibitors [143]. The mechanism of action of such isocoumarins is asserted to be reaction of the active site cysteine with the carbonyl group to form an acyl enzyme which, in some cases, may further react with another active site nucleophile to form an additional covalent bond. The inventors' objective here is apparently to expand on their original claim for this class of compounds as serine protease inhibitors [156]. Although such analogues are indeed potent serine protease inhibitors, a representative example (55) is only weakly active against calpain (10 μM) [14].



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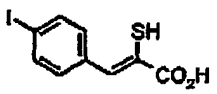
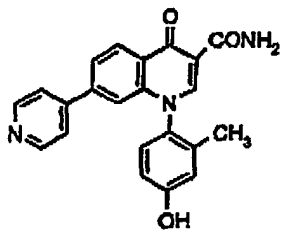


56 PD 150606

Investigators at Warner-Lambert discovered that simple mercaptoacrylic acids (e.g., 56, PD 150606; IC_{50} = 210 nM) are low molecular weight, relatively potent, selective and reversible calpain inhibitors [39]. As mentioned earlier, x-ray crystallography revealed that this inhibitor binds to the non-catalytic calcium-binding domain VI of calpain, but at a site remote from any calcium binding loop [13]. PD 150606 is cell-permeable and demonstrates neuroprotective activity in cultured cerebrocortical neurones and cerebellar slices, but possesses chemical properties which are not optimal for *in vivo* investigations.



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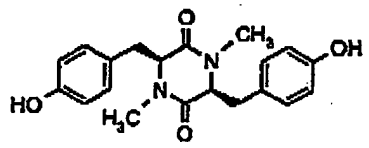
Commercially available aurintricarboxylic acid is a weak reversible cell-permeable calpain inhibitor [92]. Also, a 3-nitro-2-pyridyl disulfide derivative of a hexapeptide thiol-binding fragment of high molecular weight kininogen, a member of the cystatin superfamily, was reported to be a weak irreversible calpain inhibitor [93].



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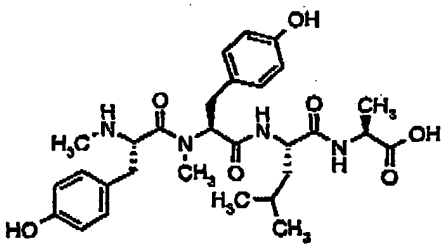
A class of moderately potent quinolone-3-carboxamide calpain inhibitors (57; IC_{50} = 510 nM) was described by Sterling Winthrop Pharmaceutical Co. [157]. These compounds are particularly interesting because they are non-peptidic and possess no obvious enzyme-reactive group, and may possibly bind to calpain at a position distinct from the active site.



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Sterling Winthrop has also reported two novel natural product calpain inhibitors isolated from an actinomycete strain of *Streptomyces griseus* [94]. The first, a diketopiperazine of N-methyltyrosine (58), displays moderately good *in vitro* activity (0.8 μM), particularly for a small molecule with no obvious enzyme-reactive group. The second, a tetrapeptide analogue, N-methyltyrosyl-N-methyltyrosyl-leucyl-alanine (59), shows somewhat lesser activity (2.1 μM). Though interesting in their own right, it is unclear whether these novel leads can progress to drug candidates. To our knowledge, no further work on these compounds has since been reported.

Cephalon very recently disclosed that novel peptidyl-2-amino-1-hydroxyalkanesulfonic acids are potent calpain inhibitors. The potency of these inhibitors (10 - 32 nM) is comparable to the analogous peptide aldehydes, but the specified compounds have the advantage of excellent aqueous solubility, which could potentially simplify *in vivo* administration [158].

5. Expert opinion

Considerable effort has been expended in studying calpain's structure, natural functions and substrates, and a diversity of synthetic inhibitors have been evaluated in the search for a viable drug candidate. These efforts have generated an enormous wealth of knowledge about the role of calpain during neurodegenerative events and have confirmed it as a prominent contributor to neuronal necrosis resulting from ischaemia, traumatic brain injury or spinal cord injury. Yet, despite the identification of inhibitors that are highly potent and bioavailable, progression of a drug candidate to clinical status has remained an elusive goal. The most potent and widely studied class of inhibitors, peptidyl and peptide mimetic aldehydes, have been valuable research tools although aldehydes in general are thought to harbour potentially significant pharmacological liabilities. Consequently, there is understandable reluctance in promoting an aldehyde to clinical trials. Despite such shortcomings, a suitably potent and selective aldehyde inhibitor, or prodrug thereof, which demonstrates a significant degree of efficacy in humans could arguably be an important milestone achievement. Currently, the most interesting candidate is MDL 28170 (**8**, Merrell) which, as discussed earlier, has been shown to be neuroprotective in rat models of focal ischaemia, even when administered up to 6 h post-infarct [40]. Whether this or some other aldehyde analogue progresses to human trials remains to be seen.

Beyond aldehydes, the next most promising class of inhibitors would appear to be the α-ketoamides, several of which are neuroprotective in rats [52,53]. The primary obstacles to further development of this class of inhibitors are their generally lower potency compared to the corresponding aldehydes, poor solubility, and the attendant formulation difficulties. For example, AK-295 (**36**, Alkermes) was administered intra-arterially or, more heroically, AK-275 (**35**, Alkermes) was administered *via* supracortical perfusion directly into the cortical surface, hardly a technique amenable to human studies. Along with the requirements for blood-brain barrier and cell membrane permeability, these hurdles have presented a formidable gauntlet for any potential drug candidate to surmount. Overall, though highly potent inhibitors are now available, improvements in bioavailability and specificity would be greatly welcomed.

Not surprisingly, since the normal roles of calpain *in vivo* are not completely understood, a significant degree of hypothesis surrounds the potential utility of calpain inhibitors to treat any of the pathological states implicated by its overactivation. The possibility that calpain plays an important, perhaps even critical, role in a number of biochemical cascades and metabolic processes in the CNS lends a degree of uncertainty regarding the net effect of inhibiting such an integral player, particularly in a chronic setting. Nonetheless, the rationale of targeting calpain is strengthened by virtue of it being a downstream event from any of the mechanisms which lead to glutamate or calcium elevations, possibly offering a common approach to the treatment of a number of neurodegenerative diseases. Calpain inhibitors would also presumably be devoid of the psychotomimetic side-effects apparently associated with NMDA receptor antagonists [95]. Nevertheless, it remains to be demonstrated in a clinical setting whether calpain inhibition during a pathological event can provide effective, practical and safe therapy for any of the degenerative processes putatively associated with its dysregulation.

The underlying assumption that inhibition of an important overactivated biomodulator such as calpain would be expected *a priori* to have some therapeutic benefit, at least for an acute condition, has been bolstered by some of the encouraging results from the *in vitro* and *in vivo* studies conducted to date. Accordingly, a proof of concept appears to be established in selected animal models. On the other hand, stroke being a highly variable condition, there remains reasonable doubt as to the relevance of any current animal model of stroke in predicting clinical efficacy [96]. The development of more relevant and reliable tissue and animal models would help to provide a deeper understanding of calpain's normal role and the consequences of its acute or chronic inhibition. For example, the availability of calpain knock-out mice (i.e., a chronic treatment model) might help to unravel the consequences of prophylactically inhibiting an enzyme which may play both important and varied roles under normal circumstances.

Researchers have also been hampered by the lack of an X-ray crystal structure of calpain's active site to refine the currently sketchy pharmacophore map, which until now has been built indirectly from SAR studies of the various classes of substrates and inhibitors. Efforts to this end have not yet succeeded, due to a variety of factors, such as the autolytic behaviour

and highly hydrophobic nature of the activated form of the enzyme, which tend to discourage crystallisation. Currently, the only available structure is of domain VI (of the 30 kDa regulatory subunit) complexed with PD 150606 (56, Warner-Lambert) [13].

Despite the impressive advances made after more than two decades of research into the characterisation, biochemistry, and pharmacology of calpain, a drug candidate has yet to reach the clinic. Indeed, there are no inhibitors of any cysteine protease on the market despite the intense efforts of numerous investigators in industry and academia. One can only surmise, as the field continues to mature with additional biological studies and the discovery of inhibitors with improved physicochemical and pharmacological properties, that we may someday anticipate a calpain inhibitor as a treatment option for ischaemia, traumatic neuronal injury, or associated neurodegenerative diseases.

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Patents

Patents of special note have been highlighted as:
- • of interest
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101. MERRELL PHARM., INC.: US5736520 (1998).
•• This patent, a continuation-in-part of an application filed in 1988, claims use of Z-Val-Phe-H (MDL 28170) as a calpain inhibitor.

102. SANOFI WINTHROP: WO9641638 (1996).

103. SUNTORY LTD.: US5081284 (1992).
• This patent, filed in 1989, claims dipeptide aldehydes including Z-Leu-Nle-H.

104. SUNTORY LTD.: EP-543510-A2 (1993).

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106. SENJU PHARM. CO. LTD.: EP-771565-A2 (1997).

107. CEPHALON, INC.: WO9721690 (1997).
•• This application discloses potent calpain inhibitors with the D configuration at P_2.

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•• Potent peptide mimetic benzothiazine aldehyde and α-ketoamide inhibitors were claimed.

129. BASF AG: WO9823581 (1998).

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132. SMITHKLINE BEECHAM CORP., CEPHALON, INC.: WO9841506 (1998).

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135. MITSUBISHI KASEI CORP.: EP-520427 (1992).

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137. CEPHALON, INC.: US5639732 (1997).

138. GEORGIA TECH. RES. CORP.: WO9212140 (1992).
•• Potent α-ketoamide and α-ketoacid calpain inhibitors as well as less potent α-ketoesters were described.

139. GEORGIA TECH. RES. CORP.: US5514694 (1996).

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151. CEPHALON, INC.: US5658906 (1997).

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